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14. ABSTRACT

The goal of this project is to demonstrate that enzymatically active prostate specific antigen (PSA) in the prostatic microenvironment can be used to locally activate either prodrugs or imaging systems. The substrate chosen was a 3 component system composed of a peptide sequence with affinity for PSA, an imaging agent and a deactivating bridge-linker, which electronically incapacitates the imaging agent. On PSA mediated release, the peptide sequence is designed to uncouple from the bridge, which then undergoes spontaneous decomposition, releasing free imaging agent The linker selected was 4-amino benzyl alcohol and proof of principle studies were conducted with a tyrosine derivative to which was coupled a series of 3 image contrast agents. The compounds underwent studies to evaluate enzymatic release on exposure to PSA, which successfully confirmed the design hypothesis.

15. SUBJECT TERMS
Chemical synthesis, prodrug, targeted chemotherapy, imaging agents, prostate specific antigen

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DAMD17-02-1-0254 SECOND YEAR REPORT

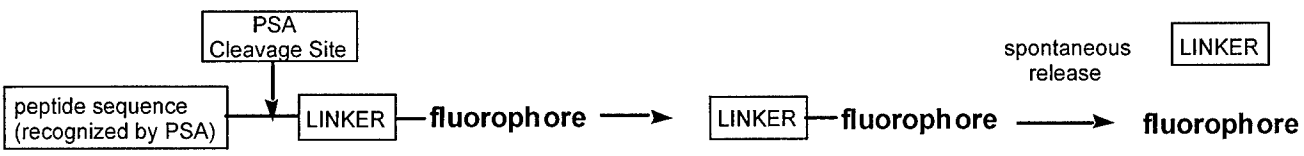
Introduction:

The overall goal of this project is to demonstrate that enzymatically active PSA in the prostatic microenvironment can be used to locally activate prodrugs and imaging systems. The research is based on the finding that PSA is enzymatically active, and has a restricted pattern of peptide bond cleavage. To demonstrate this we designed substrates for PSA that have 3 components: (1) a peptide linkage with affinity for PSA (2) a fluorophoric imaging agent and (3) a deactivating bridge, which electronically incapacitates the imaging agent until PSA activates the substrate.

Body: As outlined in the Statement of Work, the initial research plan called for design of the three component PSA substrates (objective 1), chemical synthesis of the agents (objective 2), investigation of enzymatic activation (objective 3) and subsequent biological studies and refinement (objectives 4-6). Given the difficulty encountered with chemical synthesis of the desired agents in year 1, [1] new strategies had to be investigated and considerable effort applied to objectives 1 and 2. Based on issues relating to both chemical stability and chemical reactivity, [1] we concentrated effort on a restricted set of image contrast agents and designed synthetic routes to a series of candidates that would allow us to address the fundamental proof of principle required for development of clinically useful PSA activated imaging agents. The synthesis hurdles have now been overcome, and a series of PSA substrate compounds have been prepared and were subjected to evaluation. PSA mediated release of fluorophore was confirmed in all cases, confirming the design protocol and allowing us to refine candidate compounds to now address objectives 4-6.

Rationale for image agent design

Based on the rapid clinical progression of certain prostate cancers, an imaging method capable of identifying Gleason grade ≥4 disease within the prostate gland could provide the basis for patient selection for more aggressive initial therapeutic approaches.[2] A number of image contrast enhancing agents have been studied for use in conjunction with ultrasound methods of detection with varying degrees of success.[3] However, immunohistochemical studies have also shown that Gleason grade bears an inverse correlation with the concentration of enzymatically active prostate specific antigen (PSA).[4] PSA is a serine protease;



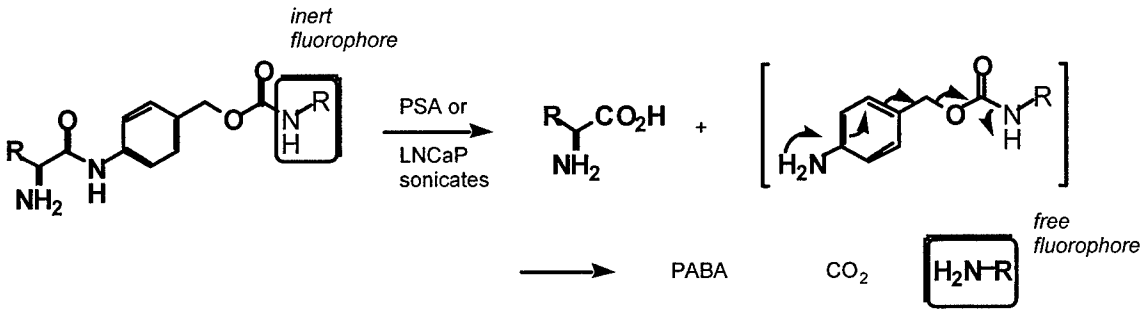
Scheme 1. Three component system for PSA activated image contrast agent

however, PSA in serum (but not in the prostatic microenvironment) is rapidly inactivated by binding to serum proteins.[5] An attractive possibility, therefore, is the design of an imaging system, which exploits the

enzymatic efficiency of PSA in the prostatic microenvironment. Our strategy was to conjugate a proteinogenic PSA substrate to a masked fluorophore via an inert spacer/linker group, such that the free fluorescent molecule is liberated on proteolysis (Scheme 1).[6]

Choice of linker

Our preferred choice for the inert linker is the *p*-aminobenzyl alcohol pioneered by Katzenellenbogen.[7] This allows coupling of peptide based enzyme substrates through the N terminus, with the alcohol group incorporated into a carbamate which masks the amino containing molecule targeted for delivery. Selective enzymatic hydrolysis of the amide group results in (a) generation an exomethylene iminium ion which is then captured by water to regenerate the free linker (b) concomitant expulsion of CO_2, rendering the reactions essentially irreversible and (c) expulsion of the free amine as shown in Scheme 2.[7] The key is to harness amino containing substrates in the system where differences in the chemistry between the carbamate and amino form are pronounced.[10] Anilino containing fluorophores were deemed excellent substrates for this system, as discernable differences in UV and fluorescence characteristics would be expected for the free aniline as opposed to the linked carbamate form. However, complications encountered during the synthesis of such agents may limit the general applicability of this approach, and judicious selection of synthetic targets is required. [1]



Scheme 2 . PSA mediated release of Tyr-linker conjugates

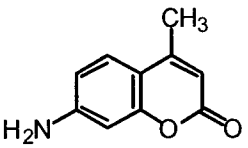
Choice of enzyme substrate

To simplify synthetic chemistry concerns, we initially elected to provide proof-of-principle with a minimal substrate and selected tyrosine conjugates for development. In addition to precedent for PSA mediated hydrolysis of tyrosyl conjugates, such derivatives would also be substrates of α–chymotrypsin, an important and well studied enzyme.[6]

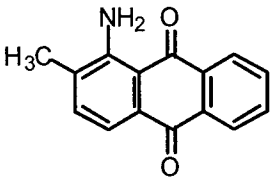
Choice of fluorophores

Our goal was to initially investigate coupling with readily available aniline based fluorophore dyes. However, given the problems encountered with luciferin derivaitves,[1] the following were selected: aminomethyl

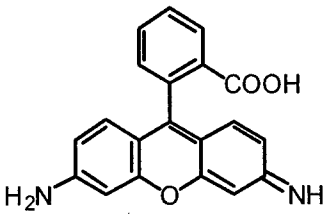
coumarin (7-AMC), disperse orange 11, and rhodamine 110. The dyes were selected on the basis that their UV characteristics are all greatly influenced by the electron donating capacity of the anilino nitrogen group.



7-AMC



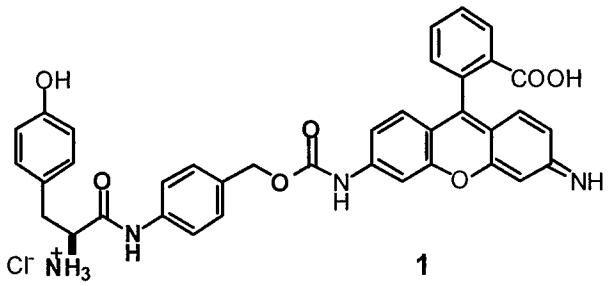
disperse orange 11



Rhodamine 110

Results of investigation

Following extensive investigation to appropriate synthetic methods, including appropriate selection of protecting groups, tyrosine conjugates **1-3** were successfully prepared. The detailed procedures and protocols employed are documented in the publication enclosed in the Appendix.[8] Key to the success of the synthesis was use of the bis-alloc masked tyrosyl derivative **4**, from which all of the systems were made accessible.



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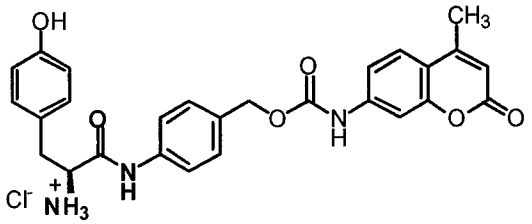

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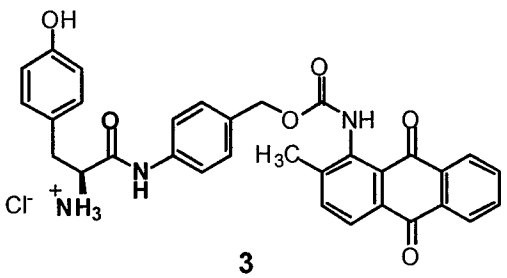

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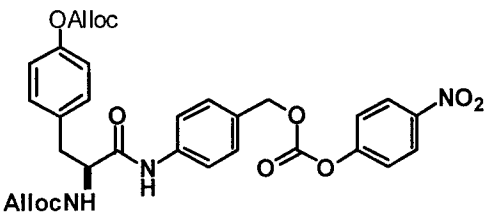

4

With three substrates in hand, spectroscopic and enzymatic studies were conducted to establish proof of concept for use as image contrast agents. Enzymatic release of the fluorophores was probed using fresh, enzymatically active PSA and chymotrypsin, using UV detection to quantitate (and fluorescence in the case of **2**). Release of fluorophore correlated with release of *p*-aminobenzyl alcohol and tyrosine, confirming the function of the self-immolative linker. As can be seen (Table 1), though proof-of-concept is established, in all of the present examples, α-chymotrypsin proved more effective than PSA at cleavage. Though this is unsurprising, more complex oligopeptide substrates are known whose specificity for PSA outranks

chymotrypsin significantly. [6] It was thus decided to elaborate the substrate with the most selectivity and activity (**2**) and to incorporate these recognition elements to it as an immediate priority.

Table 1 Enzyme mediated release of chromophores[#8]

Entry	Substrate	UV λ_{max} conjugate	UV λ_{max} free	α-chymotrypsin[a]	PSA[a]
1	**1**	300	497	9	4
2	**2**	328[b]	352[c]	24	10
3	**3**	285	486	18	6

mM/h/mg fluorophore released; b fluorescence emission λ_{max} 397; c. fluorescence emission λ_{max} 435

[#]Substrates and controls were incubated for 24-120 h at 37°C. Specific activity was determined on the basis of mM released fluorophore per unit time per unit mass of enzyme.

Key research accomplishments:

- Demonstration that self-immolative linker [4-amino benzyl alcohol] can be used to release and activate image contrast agents from enzyme substrates
- Confirmation that PSA can trigger simple tyrosyl linked substrates to release small molecules
- Efficient chemical synthesis of a family of PSA activated image contrast agents

Reportable outcomes:

Publications already in print

Image contrast agents activated by prostate specific antigen (PSA), Graham B. Jones, Longfei Xie, Ahmed El-Shafey, Curtis F. Crasto, Glenn J. Bubley and Anthony V. D'Amico, *Bioorganic and Medicinal Chemistry Letters* **2004**, *14,* 3081

Presentations

PSA Activated prodrugs as prostatic imaging agents , 227th ACS National Meeting, Anaheim, CA, March 28-April 1, 2004

Funded Grants

NIH SPORE award $1.6 million [Bubley, co-PI]

Trainees Mentored

Ahmed El-Shafey [Staff scientist - bioanalytical division, Pacific Northwestern Laboratories]
Curtis Crasto [Postdoctoral Fellow, Northeastern University]
Jude Mathews [Visiting scientist, Northeastern University]
Longfei Xie [Pfizer Research]
Jane Li [current student]
Yiqing Lin [current student]

Degrees Supported

Longfei Xie MS 2004
Ahmed El-Shafey PhD 2003

Promotions:

Graham Jones – Appointed Chair of Department of Chemistry & Chemical Biology

Conclusions:

A three component system comprised of enzyme substrate, inert linker and fluorophore has been designed and activation by chymotrypsin and PSA demonstrated. Though selectivity for PSA versus other enzymes was not attained with the present substrates, required modifications have been identified and will be introduced in the immediate future of the project.

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Appendices:

1. Pre-print of: Image contrast agents activated by prostate specific antigen (PSA), Graham B. Jones, Longfei Xie, Ahmed El-Shafey, Curtis F. Crasto, Glenn J. Bubley and Anthony V. D'Amico, *Bioorganic and Medicinal Chemistry Letters* 2004, *14*, 3081.

Image contrast agents activated by prostate specific antigen (PSA)

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Glenn J. Bubley[b] and Anthony V. D'Amico[c]

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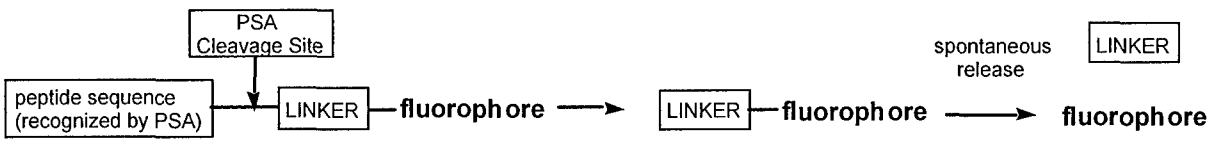
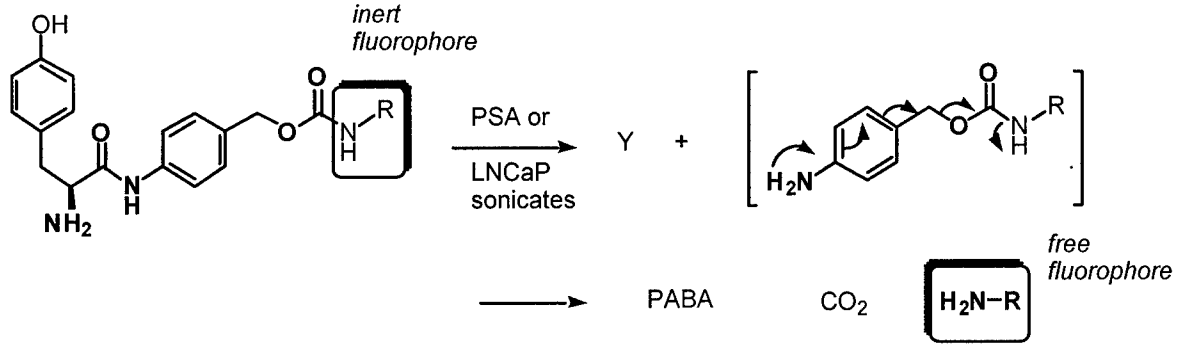
Abstract- A family of image contrast agent conjugates designed to undergo enzymatic activation has been synthesized. The agents underwent activation both with enzymatically active prostate specific antigen (PSA)and α-chymotrypsin, releasing free fluorophore via cleavage of a three component system.

Despite improvements in local therapy and increased awareness, prostate cancer continues to be second only to lung cancer as a cause for cancer deaths in men.[1] Prior investigations show that the presence of prostatectomy Gleason grade ≥4 in the radical prostatectomy specimen is the most important predictor of progression following surgery.[2] Unfortunately, the transrectal ultrasound guided sextant sampling of the prostate is subject to sampling error, and therefore biopsy Gleason grade will underestimate prostatectomy Gleason grade 4 or 5 disease in as many as 40% of men with clinically localized disease.[3] Therefore, an imaging method capable of identifying Gleason grade ≥4 disease within the prostate gland could provide the basis for patient selection for more aggressive initial therapeutic approaches.[4] Immunohistochemical studies have shown that Gleason grade bears an inverse correlation with the concentration of enzymatically active prostate specific antigen (PSA).[5] PSA is a serine protease; however, PSA in serum (but not in the prostatic microenvironment) is rapidly inactivated by binding to serum proteins.[6] An attractive possibility, therefore, would be the design of an imaging system, which exploits the enzymatic efficiency of PSA in the prostatic microenvironment. Our strategy was to conjugate a proteinogenic PSA substrate to a masked fluorophore via



Scheme 1. Three component system for PSA activated image contrast agent

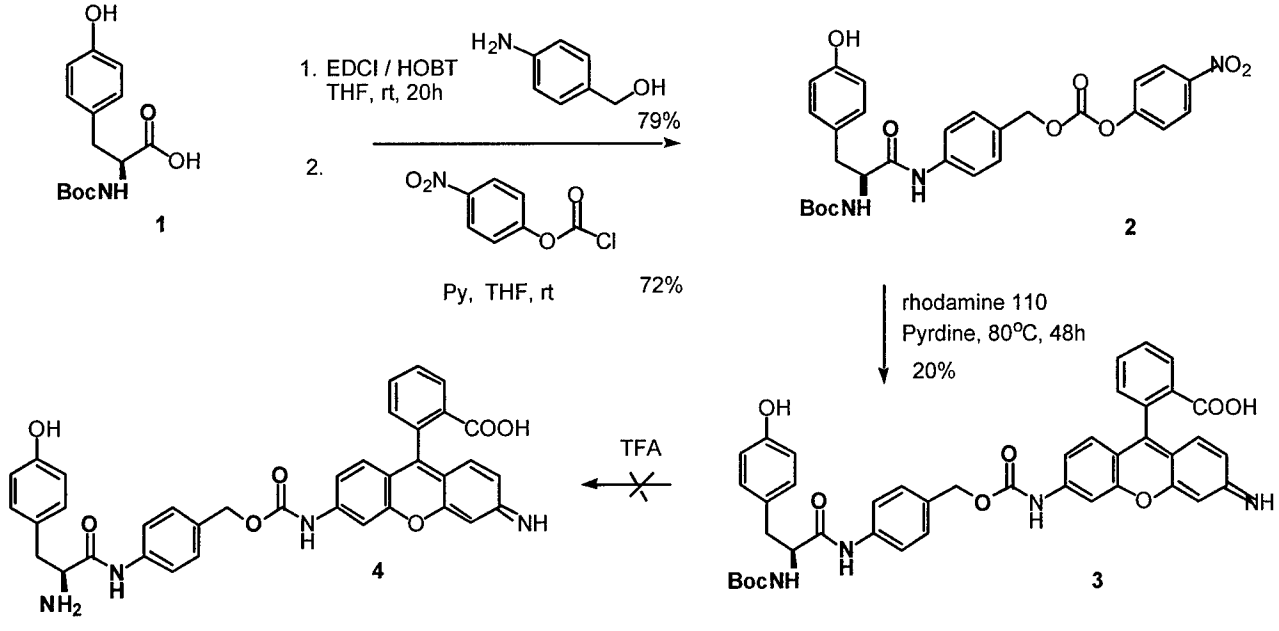


Scheme 2. PSA mediated release of Tyr-linker conjugates

an inert spacer/linker group, such that the free fluorescent molecule is liberated on proteolysis (Scheme 1).[7]

Our preferred choice for the inert linker is the *p*-aminobenzyl alcohol pioneered by Katzenellenbogen,[8] having previously employed this method for enzyme mediated cytotoxin release.[9] Though a number of high-affinity peptide substrates for PSA have been identified, we initially wished to provide proof-of-principle with a minimal substrate and selected tyrosine conjugates for examination of appropriate fluorophores (Scheme 2).[10] For our initial studies three readily available fluorophore dyes were selected - aminomethyl coumarin (7-AMC), disperse orange 11, and rhodamine 110.

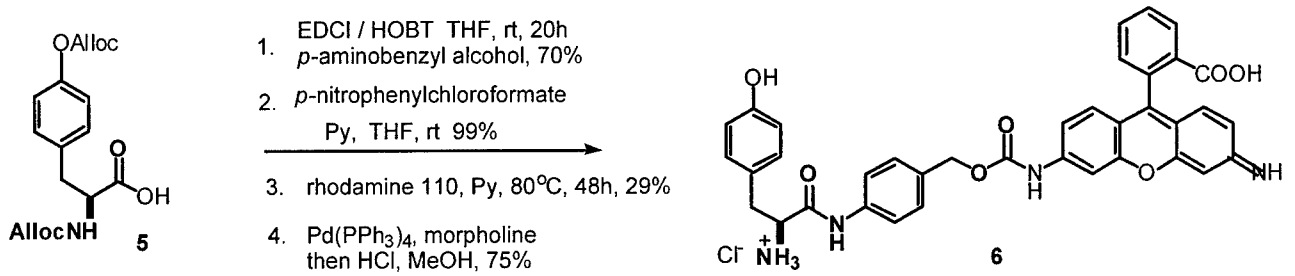
Commencing with commercially available boc-tyrosine, carbodiimide coupling with *p*-aminobenzyl alcohol,



Scheme 3. Initial route to tyrosyl rhodamine conjugate via *p*-aminobenzyl carbamate linker

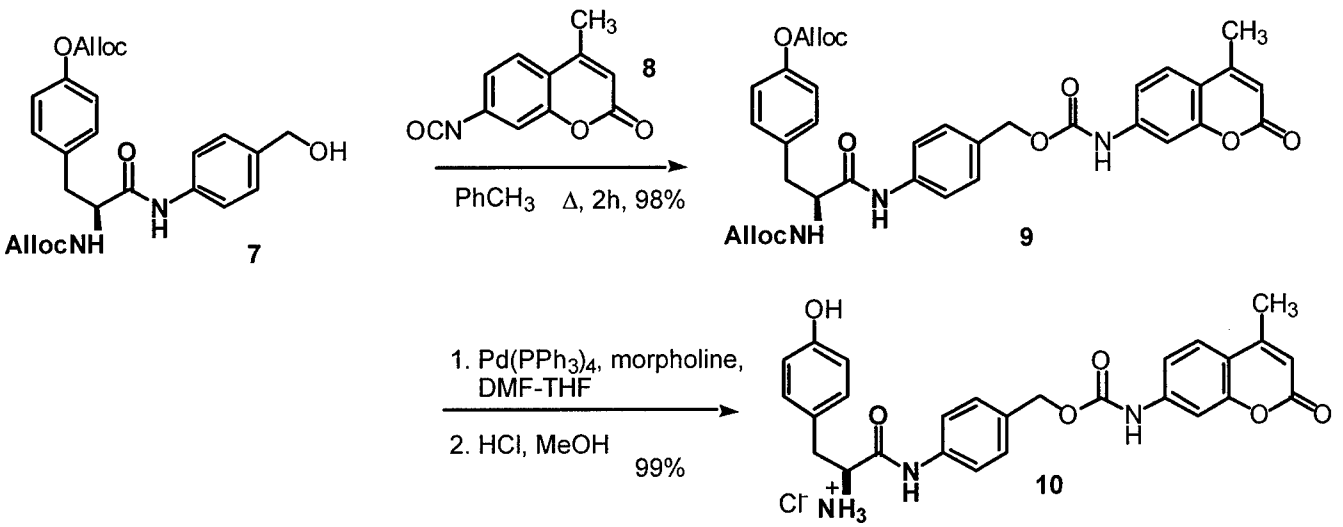
followed by reaction with *p*-nitrophenylchloroformate gave carbonate **2** without incident (Scheme 3). Coupling with free rhodamine gave **3** cleanly, albeit in low yield. However, all attempts to unmask the carbamate group resulted in decomposition of the molecule, rendering **4** unisolable. Remedy was found using the alternate bis-alloc substrate **5**, which under analogous conditions gave the intermediate carbonate, and subsequently underwent rhodamine coupling and unmasking using the Pd route, [11] to allow isolation of the

hydrochloride salt **6** in good yield (Scheme 4).



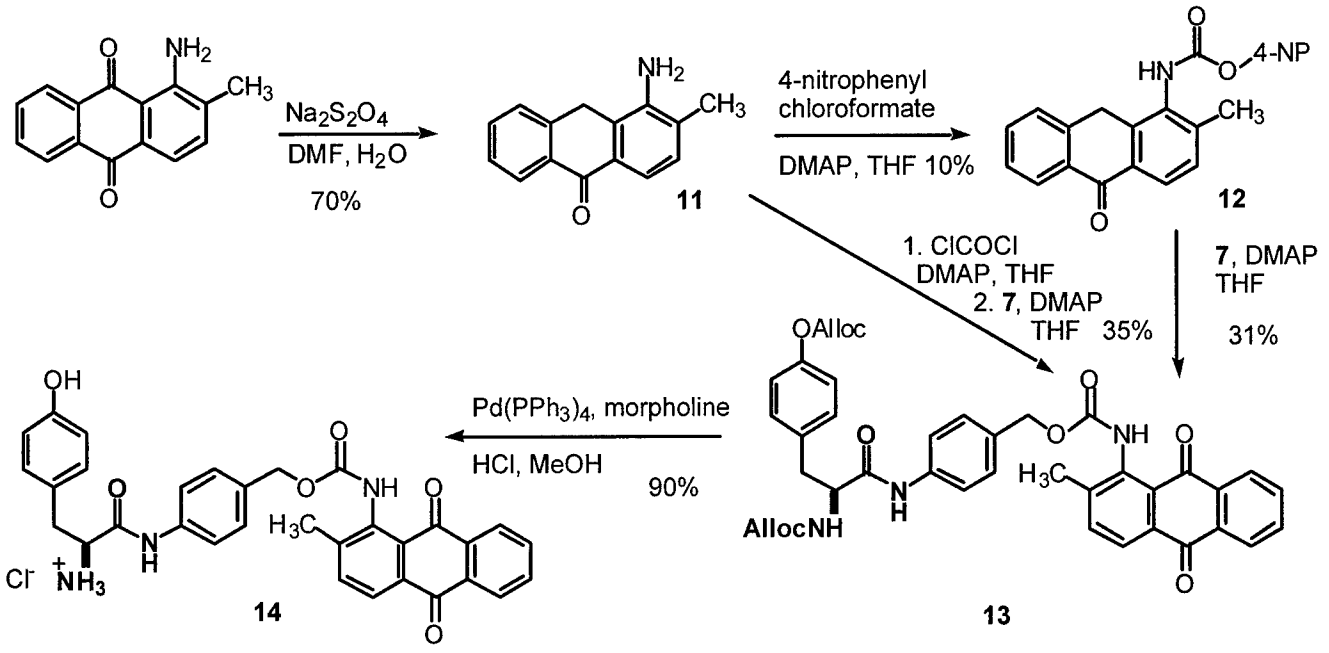
Scheme 4. Y-alloc route to tyrosyl rhodamine conjugate

With the alloc route in hand, the coumarin analog **10** was next prepared. This involved coupling of the carbonate used in Scheme 4 (**7**) with isocyanate **8** to give the masked analog **9**, which underwent clean deprotection to give **10** on workup (Scheme 5). Finally, hoping to exploit the benefits of intramolecular hydrogen bonding, the anthraquinone conjugate **14** was assembled. This necessitated selective removal of the quinone carbonyl group of aminomethylanthraquinone to allow formation of the required carbamoyl building block **11** (Scheme 6). Conversion to the *p*-nitrophenyl carbamate was inefficient, giving a complex mixture which allowed only low recovered yields of **12**. However, reaction with phosgene followed by coupling with **7** gave alloc protected adduct **13** directly, reoxidation taking place during workup. This compares favorably with the corresponding conversion of **12** to **13** and proved reliable on scale-up. Finally, unmasking allowed isolation of the hydrochloride salt of anthraquinone substrate **14** in good yield.



Scheme 5. Preparation of tyrosyl aminomethylcoumarin conjugate

With three substrates in hand, spectroscopic and enzymatic studies were conducted to determine suitability for application as image contrast agents. Enzymatic release was initially probed using fresh, enzymatically active PSA and chymotrypsin, using UV detection to quantitate (and fluorescence in the case of **10**). As can be seen (Table 1), though proof-of-concept was obtained, in the present examples, chymotrypsin is more effective than PSA at cleavage. Though this is unsurprising, more complex



Scheme 6. Preparation of tyrosyl aminomethylanthraquinone conjugate

oligopeptide substrates are known whose specificity for PSA outranks chymotrypsin significantly,[7] the most selective of these (HSSKLQ) which will now become the target of future synthetic studies.[7] Additionally, linker architecture has been shown to have a marked impact on substrate half-life in three component systems,[8,11] suggesting that specificity and stability might ultimately be tailored according to desired application. For ultimate application with *in vitro* and *in vivo* analysis it will be necessary to employ fluorophores with spectral characteristics tailored to match imaging devices. Contrast agents in the near IR range (e.g. the Cy dye family) may prove desirable,[12] and the fluorophore coupling chemistries described herein offer flexibility towards this goal.

Table 1 Enzyme mediated release of chromophores

Entry	Substrate	UV λ_{max} conjugate	UV λ_{max} free	chymotrypsin[a]	PSA[a]
1	**6**	300	497	9	4
2	**10**	328	352	**24**[b]	**10**[b]
3	**14**	272	486	**18**	**6**

a. mM/h/mg; b fluorescence λ_{max} 392 abs. 435 em.

In summary, a 3 component system comprised of enzyme substrate, inert linker and fluorophore has been designed and activation by chymotrypsin and PSA demonstrated. The results support the synthesis of more complex and selective substrates, which will be reported in due course.

Acknowledgment

We thank the Department of Defense (**DMAD17-02-1-0254**) for financial support of this work.

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